Liquid Chromatographic-Electrochemical Detection Screening Procedure for Six Nitro-Containing Drugs in Chicken Tissues at Low ppb Level

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A screening procedure is described for the detection of furazolidone, nitrofurazone, aklomide, zoalene, nitromide, and sulfanitran residues in a single extract of chicken liver, breast, or thigh muscle at the low ppb level. The method includes extraction of tissue with chloroform–ethyl acetate–dimethyl sulfoxide (50 + 50 + 0.8), adsorption on neutral alumina, and subsequent elution of the residues with pH 6.0 phosphate buffer–methanol (1 + 1). Eluants are separated on a 25 cm, 5 μm C_{18} column with pH 6.0 phosphate buffer–methanol (57.5 + 42.5) as mobile phase. The drugs are detected with an electrochemical detector in the reductive mode at −0.8 V. Mean recoveries from all tissues ranged from 76.5% for nitrofurazone to 97.1% for zoalene.

Because a wide variety of veterinary drugs are used as feed additives to promote growth and/or to prevent microbial infections in poultry (1), federal regulations have established tolerance levels for residues of the drugs in edible tissues (2). For the most part, analytical methods are available for detecting residues of individual drugs or combinations of drugs in tissues at or near the tolerance levels. In many cases, however, the methods are nonspecific, lack the necessary sensitivity, or are too lengthy to be employed routinely by the regulatory agencies in their monitoring of the nation's poultry supply. Because of these limitations of the available methodology, the Food Safety Inspection Service of the U.S. Department of Agriculture has emphasized the need for rapid, multiresidue screening procedures that are capable of detecting and tentatively identifying potential drug residues of specific chemical classes in poultry tissues at or near the tolerance levels.

Among the drugs administered to chickens are a group of antibacterial and anticoccidial drugs. They are characterized, chemically, by the presence of nitro groups and include nitrofurans, nitrobenzamides, sulfanitran, and nicarbazin. Previously, independent rapid procedures were presented for the detection and quantitation of zoalene (3) and nicarbazin (4), drugs with relatively high residue tolerance levels. Preliminary studies revealed that while those procedures were capable of detecting other nitro-containing residues in chicken tissues, they lacked sensitivity to several of the drugs with zero tolerance levels. The present study was undertaken to modify the existing procedures so that those potential residues could be detected.

METHODS

Reagents and Apparatus

(a) *Solvents.*—Ethyl acetate and methanol (distilled in glass, Burdick and Jackson, Muskegon, MI 49442); chloroform, Baker analyzed reagent (J. T. Baker, Inc., Phillipsburg, NJ 08665); dimethyl sulfoxide (DMSO) (Fisher Scientific Co., West Haven, CT 06516); and *N,N*-dimethylformamide (DMF) (Aldrich Chemical Co., Inc., Milwaukee, WI 53201).

(b) *Drugs.*—Aklomide, nitromide, zoalene, and sulfanitran (Salsbury Laboratories, Charles City, IA 50616); furazolidone and nitrofurazone (Norwich-Eaton Pharmaceuticals, Norwich, NY 13815).

(c) *Tissue homogenizer.*—Polytron (Brinkmann Instruments, Inc., Westbury, NY 11590).

(d) *Centrifuge.*—Refrigerated IEC Centra-7R. Rotor No. 822A (International Equipment Co., Div. of Damon Corp., Needham Heights, MA 02194).

(e) *Sand.*—Purified, Washed and Ignited for Boats (J. T. Baker, Inc.). Wash 60 g sand with 4–50 mL portions of methanol in 60 mL coarse fritted glass Buchner funnel. Dry 2 h at 130°C.

(f) *Neutral alumina.*—Brockman Activity I, 80–200 mesh (Fisher Scientific Co.). Insert 6 mm round glass bead into 5 mL pipet tip (Rainin Instrument Co., Woburn, MA 01801). Layer glass bead with 0.5 cm washed sand and 3.5 cm bed of neutral alumina, firmly packed (to height of 3.0 cm) by gently tapping top of pipet tip. Add 0.2 cm layer of sand. Wash column with two 2 mL portions of $CHCl_3$–ethyl acetate (1 + 1) before use.

(g) *Liquid chromatograph (LC)–electrochemical detection (ECD).*—Altex Model 100A pump (Altex Scientific, Inc., Berkeley, CA 94710) connected to BAS Model LC-4B amperometric detector (Bioanalytical Systems, Inc., West Lafayette, IN 47905): glassy carbon electrode −0.8 V vs Ag/AgCl, 20 nA full scale. Altex Model 210 A sampling valve with 50 μL loop. Recorder: Fisher Recordall Series 5000 at 10 mV full scale and chart speed 1 cm/min. Column: 25 cm × 4.6 mm id 5 μm Supelcosil LC-18 (Supelco, Inc., Bellefonte, PA 16823). Mobile phase: pH 6.0 phosphate buffer (0.05M monobasic potassium phosphate solution containing 0.001 mole EDTA adjusted to pH 6.0 with 1N NaOH)–

Table 1. Residue tolerance levels and recovery of drugs from fortified chicken tissues

Drug	Tolerance ppm[a]	Drug added, ppm	Recovery, %[b] Liver	Thigh	Breast
Furazolidone	0	0.006–0.20	89.9 ± 5.8	88.6 ± 6.8	89.4 ± 4.6
Nitrofurazone	0	0.006–0.20	74.0 ± 5.7	78.7 ± 4.8	76.8 ± 6.2
Aklomide	3.0–4.5[c]	0.02–10.0	95.2 ± 3.3	96.3 ± 3.9	98.5 ± 3.9
Zoalene	3.0–6.0[c]	0.02–10.0	96.2 ± 2.2	95.9 ± 3.8	99.2 ± 3.1
Nitromide	0[c]	0.005–0.08	90.8 ± 4.8	82.1 ± 5.6	93.0 ± 3.9
Sulfanitran	0	0.025–0.50	96.9 ± 4.1	97.0 ± 4.0	91.7 ± 5.6

[a] Reference 2.
[b] Mean and standard deviation of 3 determinations at 5 or 6 serial concentrations. Based on analysis of extract equivalent to 1.875 g tissue.
[c] Includes metabolites.

methanol (57.5 + 42.5) purged with helium. Elute samples isocratically at 1.0 mL/min. Sampling technique: Purge 2 mL sample in 9 mL vial with helium (saturated with mobile phase) for 2 min to exclude oxygen. Draw 0.6–0.8 mL sample through capillary tubing into loop, by way of vent, with hypodermic syringe inserted in sampling valve in load position.

Determination

Place 2.5 g frozen, ground tissue into 50 mL polypropylene centrifuge tube. Let sample partially thaw. Add 20 mL $CHCl_3$–ethyl acetate–DMSO (50 + 50 + 0.8) and blend 45 s with tissue homogenizer at medium speed. Centrifuge 5 min at 3500 rpm (10 min for liver tissue). Remove and discard any aqueous layer. Recover solvent, filter it through small plug of glass wool packed tightly in 4 mL disposable Pasteur pipet, and collect filtrate. Pass 15 mL filtrate through neutral alumina column. (If volume of recovered filtrate is < 15 mL, record actual volume and add $CHCl_3$–ethyl acetate (1 + 1) to 15 mL. Calculate recoveries accordingly.) Wash sides of column with 6 mL $CHCl_3$ in 1.0, 1.0, 1.0, and 3 mL increments. Remove excess $CHCl_3$ from column with air pressure and maintain pressure until column dries as evidenced by disappearance of moisture on outside of column. Continue air pressure for additional 5 min. Elute column with 0.05M pH 6.0 phosphate buffer (less EDTA)–methanol (1 + 1); collect first 2 mL effluent in 2 mL glass-stopper volumetric flask. Shake stoppered flask thoroughly, and use disposable Pasteur pipet to transfer effluent to 9 mL screw-cap specimen vial. Inject 50 μL sample onto LC column according to procedure described above. Perform all phases of analytical method in absence of direct lighting.

Recovery Studies

Fortify 2.5 g drug-free, frozen, ground tissues by injection with 5 μL methanol (aklomide, nitromide, zoalene, sulfanitran) or DMF solutions (furazolidone, nitrofurazone) of drugs at 5 or 6 concentrations. Hold fortified samples at −20°C overnight (approximately 18 h) before analyses. Use unfortified tissues as controls.

Recovery data were determined by comparing peak heights or areas with standards. Preliminary studies had shown that a well-conditioned column (5–7 days with mobile phase at 1 mL/min) is required to avoid excessive tailing of the nitrofuran compounds, especially furazolidone.

Results and Discussion

The extraction procedure eventually employed in this study is a modification of the previously published procedure for nicarbazin residues in chicken tissues (4). Because of the tolerance levels set for nitromide, sulfanitran, furazolidone, and nitrofurazone (Table 1), the volume (15 mL) of the $CHCl_3$–ethyl acetate–DMSO extract and, consequently, the size of the alumina column (3 cm) needed to be larger than those used for nicarbazin. Preliminary studies that employed a similar modification of the zoalene procedure (3) provided adequate recoveries but resulted in asymmetrical LC peaks for several of the drugs, as a result presumably of the pH 3.5 formate buffer in the mobile phase.

Figure 1 depicts LC–ECD chromatograms of extracts of liver and thigh muscle fortified with 5 ppb furazolidone and nitrofurazone; 10 ppb aklomide, zoalene, and nitromide; and 12.5 ppb sulfanitran and their control extracts at a potential of −0.8 V and attenuation of 20 nA full scale. Typical chromatograms of fortified and unfortified breast tissue are the same as those for thigh muscle. Furazolidone and nitrofurazone do not separate under the LC conditions employed. Furazolidone (Rt 4.0 min) eluted just prior to nitrofurazone (Rt 4.1 min). Partial separation, sufficient for tentative identification, can be achieved by increasing the ratio of pH 6 buffer to methanol. However, changing the ratio even slightly results in marked changes in the retention time of sulfanitran and, thereby, increases the time of analysis and decreases detection at the lowest possible level for sulfanitran, as a result of peak broadening. Since the procedure is designed for rapidly screening the 6 drugs, the time of analysis and detection at the lowest possible level outweigh the advantage of tentatively identifying the co-chromatographing residues initially. As stated above, the compounds can be tentatively identified by altering the mobile phase.

An alternative procedure for distinguishing furazolidone and nitrofurazone is to use the well-known tendency of nitrofurans to undergo photochemical reactions (5, 6). After brief exposure to sunlight or 15 min exposure to 450 ft-candles of fluorescent light, liquid chromatography of exposed nitrofurazone solutions results in the appearance of a second peak at 3.7 min with a corresponding decrease of the parent nitrofurazone peak. Furazolidone, however, remains a single peak at 4.0 min. The persistence of a single peak does not imply that furazolidone is resistant to photochemical reactions but that the parent drug and its reaction product co-chromatograph under the LC conditions in this procedure. Chromatographic studies on exposed furazolidone solutions that use a mobile phase of pH 6 buffer–methanol (7.5 + 2.5) revealed a peak at 8.5 min for the parent drug and 8.0 min for its photochemical reaction product. Corresponding retention times for nitrofurazone and its reaction product in this mobile phase were 8.9 and 7.9 min, respectively.

Under the LC conditions of the present study, a consistent background peak at the retention time of furazolidone and nitrofurazone (equivalent to 0.5 ppb) limits the lowest detectable level (LDL) of these drugs to 2.5 ppb (5 × background). A contaminant, of variable concentration, present only in liver (Rt 4.3 min) does not interfere with the detection

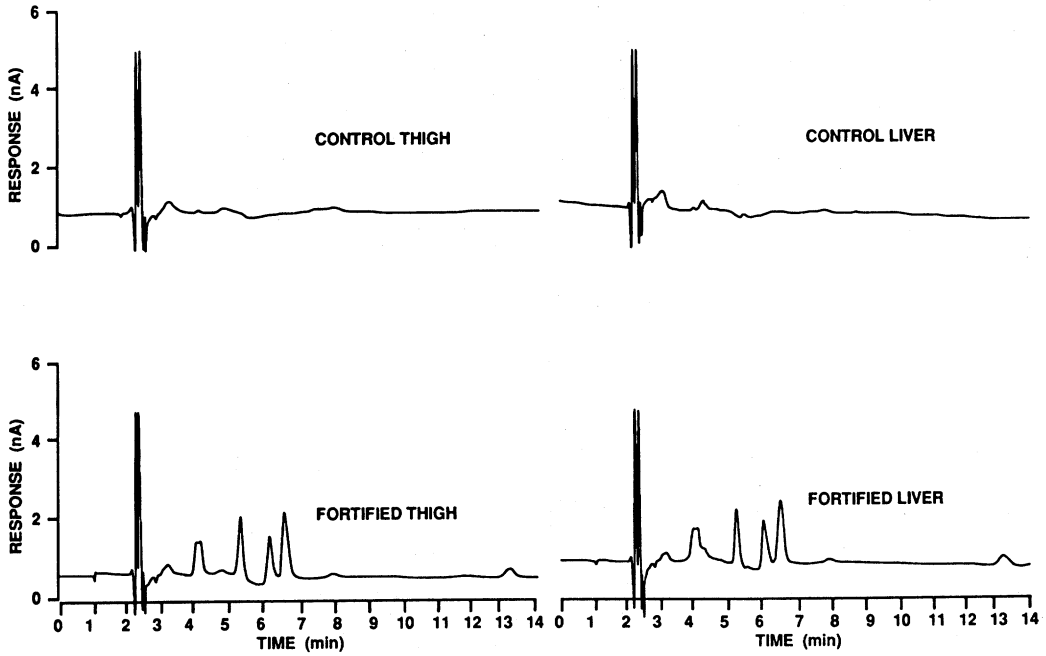

Figure 1. LC chromatograms of extracts of liver and thigh muscle fortified with 5 ppb furazolidone (Rt 4.0 min) and nitrofurazone (Rt 4.1 min); 10 ppb aklomide (Rt 5.3 min), zoalene (Rt 6.2 min), and nitromide (Rt 6.7 min); 12.5 ppb sulfanitran (Rt 13.2 min) and their control extracts at a potential of −0.8 V and attenuation of 20 nA full scale.

of furazolidone and appears as a shoulder on the nitrofurazone peak. Subsequent studies have shown that the contaminant is all but absent in fresh, frozen liver tissues but increases in concentration during frozen storage. A noncontaminating peak at Rt 5.5 min performs similarly. Liver tissue must, therefore, be analyzed as soon as practical to prevent the contaminants from obscuring relevant peaks.

No contaminating peaks interfered with the detection and quantitation of aklomide, zoalene, nitromide, or sulfanitran at an attenuation of 20 nA full scale under the LC conditions of this study. LDLs (2 × noise) for these drugs were < 2 ppb for aklomide, zoalene, and nitromide and < 6 ppb for sulfanitran. Lower LDLs for all individual drugs can be attained by slightly altering the mobile phase (decreasing the ratio of pH 6 buffer to methanol) and/or by increasing detector sensitivity.

Although the modified procedure quantitatively extracts the dinitrocarbanilide portion of the nicarbazin complex from chicken tissue, the LC conditions employed are not conducive for detecting this drug (Rt 90 min), and the previous procedure is recommended.

Table 1 summarizes recovery studies performed on varying concentrations of the drugs from fortified liver, breast, and thigh muscle. Detector response (peak height) at the range of concentrations in this study were linear for each drug ($r > 0.9995$). Except for nitromide, recoveries of the individual drugs are consistent between tissues. No satisfactory explanation for the lower recovery of nitromide from thigh muscle was forthcoming from a series of experiments. An interaction appears to occur however, between the $CHCl_3$–ethyl acetate–DMSO extractables of thigh muscle and nitromide.

Using the procedure outlined here, an analyst can concurrently prepare 2 tissue extracts for liquid chromatography every 75 min; LC analyses can be performed at 15 min intervals since no detectable late eluting peaks have been observed in these studies. Nevertheless, occasional washing (biweekly) of the column with pH 6.0 buffer–methanol (30 + 70) is recommended to limit peak broadening.

Work is currently in progress to apply this technique to incurred tissue samples to study the extraction efficiency from such tissues, detection of reported and unreported metabolites, and residue depletion from tissues following withdrawal of birds from the medicated feeds.

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